

-4-

- b) bringing said SV40-like particles obtained, in step (a) into contact with said exogenous antisense RNA, or ribozyme RNA, or RNA or substantially histone-free DNA which inhibits or prevents the expression of undesired proteins in a mammalian cell, to give *in vitro* constructed SV40 pseudoviruses.
37. (Twice Amended) A method according to Claim 35 wherein in step (a) at least one other SV40 protein, preferably SV40 agnoprotein, is added to the mixture of SV40 capsid protein or proteins and the exogenous antisense RNA or ribozyme RNA or RNA or substantially histone-free DNA.
43. (Twice Amended) A method of transforming a substantially histone free DNA, RNA, antisense RNA, ribozyme RNA, protein or peptide product into a cell comprising infecting said cell with the construct of Claim 1.
45. (Twice Amended) A composition comprising an effective amount of the complex of Claim 1 in a pharmaceutically-acceptable carrier.
46. (Twice Amended) A composition comprising an effective amount of the infected cells according to Claim 41, in a pharmaceutically-acceptable carrier.

Amendments to the claims are indicated in the attached "Marked Up Version of Amendments" (pages i - iii).

REMARKS

Claims 1-2, 4-13, 16-20, 22-37, 41-43 and 45-46 are currently pending in the application. Claim 44 is cancelled, and Applicants reserve the right to pursue this claim in a continuing or other application. Claims 1, 6-7, 18, 20, 35, 37, 43 and 45-46 are amended. The amendments find support in the specification and claims as filed, and such support is discussed in the relevant sections below. No new matter is added by these amendments.

Applicants' representatives, Doreen M. Hogle and Joyce C. Hersh, thank the Examiner for the telephonic discussion of April 18, 2001, in which the outstanding rejections were

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discussed. The claim amendments proposed during that discussion have been made, and are explained below.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 1-2, 4-8, 10-13, 16-20, 22-26, 28-37 and 41-46 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification in such a way as to enable one of ordinary skill in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The Examiner reiterates that the specification "makes clear the necessity of having an *ori* sequence in each nucleic acid which is encapsulated in the claims SV40 protein capsid structures."

Applicants provide herewith a Declaration under 37 U.S.C. § 1.132, by Orly Ben-Nun Shaul, and executed February 4, 2001, providing data showing that the *ori* sequence is not necessary for packaging.

Applicants respectfully request that the rejection on this basis be reconsidered and withdrawn.

Claims 43-44 also stood rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification in such a way as to enable one of ordinary skill in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

While not agreeing with the Examiner, solely to speed prosecution, Applicants are willing to cancel or amend claims 43-44. In the telephonic conversation of April 18, 2001, Applicants agreed to cancel claims 43-44. In the present Reply, however, Applicants have cancelled Claim 44, and offer an amended Claim 43 that they sincerely hope will obviate the rejection. Should the Examiner disagree or if substantive examination is required to review the amendments, Applicants representatives are willing to discuss further amendments or cancellations.

Applicants respectfully request that in view of the amendment of Claim 43 and the cancellation of Claim 44, the rejection on this basis be reconsidered and withdrawn.

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Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 1-2, 4-13, 16-17 and 34 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

The Examiner states that Claim 1, from which the other claims depend, contains an internal inconsistency in that section “e)” provides a constituent that is an “exogenous protein or peptide product”, and that there the “DNA construct” of the preamble may be left without an element of DNA.

Applicants note that the second half of Claim 1 recites that the complex further comprises operatively linked elements, which would provide for the “DNA” of the DNA construct.. However, Applicants have amended Claim 1 as discussed on April 18, 2001, and have deleted “DNA” from the preamble. Applicants respectfully submit that the claims as amended are clear on their face, and respectfully request that the rejection on this basis be reconsidered and withdrawn.

Claims 45-46 were also rejected, the Examiner believing that the term “active ingredient” reads more broadly than is understood by those in the pharmaceutical arts, and that the term is vague and indefinite.

Although not specifically discussed in the telephonic conversation of April 18, 2001, Applicants have amended claims 45-46 to delete this phrase.

Claim Rejections Under 35 U.S.C. § 102(b)

Claims 1-2, 4-7, 9-10-12, 16-20, 22-25, 27-34 and 41-42 are rejected under 35 U.S.C. § 102(b), in view of Christensen *et al.* (of record) and also in view of Colomar *et al.* (of record). Applicants argued in the Reply to the previous Office Action that the DNA of Christensen *et al.* was “nucleoprotein”, not naked DNA. The Examiner noted that “naked DNA” was not claimed, and that the anticipatory rejection could be avoided by addition of such a limitation.

A key element in the present invention is the packaging of substantially histone-free DNA, *i.e.*, DNA without histones. In contrast, the particles produced by Colomar *et al.* contained histones. The absence of histones renders the constructs of the invention novel over Colomar *et al.*

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Colomar *et al.* used (page 2784, first paragraph) “purified supercoiled polyoma virus DNA” and incubated this DNA with “100-fold molar excess of completely dissociated SV40 as for the analysis shown in Fig. 4”, that is in 1M salt solution. This is the standard procedure for dissociating histones from DNA, as the mixture contained histones that were originally present in the SV40 before dissociation. As explained by Colomar *et al.* (page 2782, second column, second paragraph), the ionic strength was increased to 1M salt in order “to dissociate the DNA and protein subunits of disrupted SV40.” These protein subunits are the histones which remained associated with the DNA as nuclear protein (NP) complexes, in the particles disrupted at 0.15 M NaCl (see Fig. 1). As expected, at 1M salt, the NP complexes were fully dissociated. As shown by the authors (Fig. 4a), the physical properties of the DNA were identical to those of free, naked DNA. Therefore the “completely dissociated SV40” mixture contained SV40 capsid proteins, SV40 DNA and free histones.

Colomar *et al.* then added free polyoma DNA, anticipating that part of the polyomavirus DNA will “become associated with histones and SV40 capsid proteins” (page 2784, first column, end of first paragraph, emphasis added). In order to reconstitute the particles, they then lowered the salt concentration by stepwise addition of buffer without any salt. As shown by the authors, this procedure is “based on a method previously shown to reconstitute nucleosome core particles from histones and DNA” (page 2779, last two lines, emphasis added). Thus, their reconstituted particles contained DNA complexed with histone proteins. The process is described in detail, for the reconstituted SV40 particles, in the Discussion section (page 2785, third paragraph). The same process was used for encapsidation of polyomavirus DNA. Thus, although purified supercoiled DNA was added to the disrupted SV40, NP complexes were formed with the histones present in the incubation mixture. It is therefore clear that the re-assembled virus particles did contain histones and were not histone-free.

Applicants have amended Claim 1 to recite that the DNA is “substantially histone free”. Support for this amendment can be found at page 33, line 12 of the specification. Applicants respectfully submit that the claims, as amended, are free of the cited art, and respectfully request that the rejection on this basis be reconsidered and withdrawn.

Claims Rejections Under 35 U.S.C. § 103(a)

Claims 1-2, 4-13, 16-20, 22-37 and 41-46 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Christensen *et al.* or Colomar *et al.*, each in view of Carswell *et al.* (of

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record), Oppenheim *et al.* (*J. Virol.*, 1992, of record), U.S. Pat. No. 5,863,541, Szczylik *et al.* (of record).

The particles that Colomar *et al.* reconstituted with polyomavirus DNA were not infective. The authors state (page 2784, first column, last 3 lines) that "[the infectivity] was found to be low, about the same as that of naked polyomavirus DNA (unpublished results). SV40 can bind to and enter mouse cells, so the reason for this low infectivity is unclear." From this statement it is clear that no infective polyomavirus particles were produced. Therefore, Colomar *et al.* does not teach how to produce particles with heterologous DNA that facilitates gene delivery into cells.

Even if Colomar *et al.* were successful, their products would have been inappropriate for transfer of DNA into cells, and therefore useless. In contrast, see "transfer" of DNA at page 2, line 1 and page 3, line 25 through page 4, line 5 of the specification, and "infected cells" at page 8, line 17. The putative infective particles with heterologous DNA would be produced in admixture with reconstituted SV40. As both particles are of the same size, density and surface proteins, they would be non-separable from each other. Thus the hypothetical infectious particles produced by the method of Colomar *et al.* would always be contaminated by SV40. The problem of contamination of SV40 particles is solved by the invention described herein. No SV40 DNA is present in the complexes, as the invention is based on the utilization of recombinant SV40 proteins.

Applicants therefore respectfully submit that the claims as amended are patentable in view of either Christensen *et al.* or Colomar *et al.*, and therefore patentable in view of Carswell *et al.*, Oppenheim *et al.*, U.S. Pat. No. 5,863,541, or Szczylik *et al.* Applicants therefore respectfully request that the rejection on this basis be reconsidered and withdrawn.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If

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the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned at (781) 861-6240.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By 

Joyce C. Hersh

Registration No. 42,890

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Lexington, Massachusetts 02421-4799

Dated: *May 9, 2001*

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MARKED UP VERSION OF AMENDMENTS

Claim Amendments Under 37 C.F.R. § 1.121(c)(1)(ii)

1. (Thrice Amended) A [DNA] complex comprising semi-purified or pure SV40 VP1 capsid protein or a mixture of VP1 and at least one other SV40 capsid protein; and a constituent selected from the group consisting of:
 - a) an exogenous substantially histone-free DNA, or an exogenous substantially histone-free DNA encoding an exogenous protein or peptide product, or an exogenous substantially histone-free DNA encoding RNA;
 - b) a vector comprising any of the exogenous substantially histone-free DNAs of a);
 - c) an exogenous RNA, or an exogenous RNA encoding an exogenous protein or peptide product;
 - d) a vector comprising any of the exogenous RNAs of c);
 - e) an exogenous protein or peptide product; or
 - f) antisense RNA, ribozyme RNA or any RNA or substantially histone-free DNA which inhibits or prevents the expression of undesired protein or proteins in said mammalian cell;and further comprising operatively linked elements sufficient for one or more of the following:
 - (i) replication of said constituent;
 - (ii) expression of said constituent; and
 - (iii) prevention of expression of said undesired protein or proteins;in said mammalian cell.
6. (Thrice Amended) A complex according to Claim 1 wherein said constituent is:
 - (a) exogenous circular or linear substantially histone-free DNA;
 - (b) exogenous circular or linear substantially histone-free DNA encoding a protein or peptide product; or
 - (c[d]) exogenous circular or linear substantially histone-free DNA encoding RNA.

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7. (Thrice Amended) A complex according to Claim 6 wherein said substantially histone-free DNA is DNA which encodes a protein or peptide product, wherein said protein or peptide product is not made or contained in said cell prior to infection with the construct, or is substantially histone-free DNA which encodes a protein or peptide product, wherein said protein or peptide product is made or contained in said cell in an amount insufficient for proper cell function prior to infection with the construct, or is substantially histone-free DNA which encodes a protein or peptide product, wherein said protein or peptide product is made or contained in said cell in a form inadequate for proper cell function prior to infection with the construct, or encodes a RNA.
18. (Thrice Amended) A method for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous substantially histone-free nucleic acid comprising the following steps:
- a) allowing a semi-purified or pure SV40 VP1 capsid protein or a mixture of VP1 and at least one other SV40 capsid protein to self-assemble into SV40-like particles; and
 - b) bringing the SV40-like particles assembled in step (a) into contact with said exogenous substantially histone-free nucleic acid to give *in vitro* constructed viruses, or into contact with a vector comprising said exogenous substantially histone-free nucleic acid to give pseudoviruses.
20. (Twice Amended) A method according to Claim 18 wherein in step (a) at least one other SV40 protein, preferably SV40 agnoprotein, is added to the mixture of said SV40 capsid protein or proteins and said exogenous substantially histone-free nucleic acid.
35. (Thrice Amended) A method for the *in vitro* construction of SV40 pseudoviruses comprising exogenous antisense RNA, or ribozyme RNA or RNA or substantially histone-free DNA which inhibits or prevents the expression of undesired protein or proteins in a mammalian cell, comprising the following steps:
- a) allowing a semi-purified or pure SV40 VP1 capsid protein or a mixture of VP1 and at least one other SV40 protein to self assemble into SV40-like particles and

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- b) bringing said SV40-like particles obtained, in step (a) into contact with said exogenous antisense RNA, or ribozyme RNA, or RNA or substantially histone-free DNA which inhibits or prevents the expression of undesired proteins in a mammalian cell, to give *in vitro* constructed SV40 pseudoviruses.
37. (Twice Amended) A method according to Claim 35 wherein in step (a) at least one other SV40 protein, preferably SV40 agnoprotein, is added to the mixture of SV40 capsid protein or proteins and the exogenous antisense RNA or ribozyme RNA or RNA or substantially histone-free DNA.
43. (Twice Amended) A method of transforming [providing] a substantially histone free DNA, RNA, antisense RNA, ribozyme RNA, protein or peptide product into a cell comprising infecting said cell with [to a patient in need of such product by administering to said patient an effective amount of] the construct of Claim 1.
45. (Twice Amended) A composition comprising [as an active ingredient] an effective amount of the complex [construct] of Claim 1 in a pharmaceutically-acceptable carrier.
46. (Twice Amended) A composition comprising [as an active ingredient] an effective amount of the infected cells according to Claim 41, in a pharmaceutically-acceptable carrier.

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PATENT APPLICATION
Attorney's Docket No 2663.1003-000
Expedited Procedure under 37 C.F.R. 1.116
Examining Group 1636

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Ziv Sandalon, Ariella Oppenheim and Amos Oppenheim
Application No.: 09/068,293 Group: 1636
Filed: May 6, 1998 Examiner: W. Sandals
For: IN VITRO CONSTRUCTION OF SV40 VIRUSES AND
PSEUDOVIRUSES

CERTIFICATE OF FACSIMILE TRANSMISSION	
I hereby certify that this correspondence is being facsimile transmitted to the United States Patent and Trademark Office.	
on <u>5-9-01</u>	<u>Melissa Restolano</u>
Date	Signature
<u>Melissa Restolano</u>	
Typed or printed name of person signing certificate	

TRANSMITTAL OF
DECLARATION OF ORLY BEN-NUN-SHAUL, Ph.D., UNDER RULE 132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please find enclosed herewith an executed Declaration of Orly Ben-Nun-Shaul, Ph.D., Under Rule 132 for filing in the above captioned application.

This Declaration is being provided to support arguments which were advanced by the Applicants in Amendment B, which was mailed to the U.S. Patent and Trademark Office on November 3, 2000, in response to the Office Action mailed May 5, 2000.

Also enclosed herewith are two references, Kane, S.E. *et al.* (1988, *Mol. Cell. Biol.* 8:3316-3321) and Schreiber, E., *et al.* (1989, *Nucl. Acids Res.* 15:6419-6436). These references are mentioned in the Declaration.

09/068,293

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Please charge Deposit Account No. 08-0380 for any additional fees that may be due in this matter.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By 

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Date:

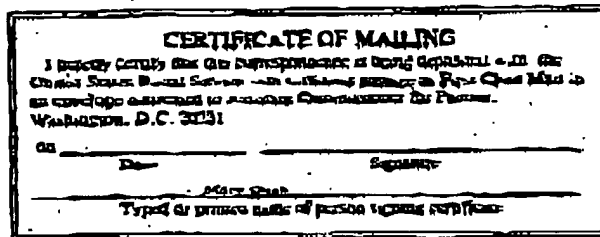
May 7, 2001

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DMH/CH/mq
November 2, 2000

PATENT APPLICATION
Attorney's Docket No. 2563 1003-400
(Serially AEM96-01A)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Ziv Sandalan, Ariella Oppenheim and Amos Oppenheim
Application No: 09/068,293 Group: 1636
Filed: May 6, 1998 Examiner: W. Sandals
For: IN VITRO CONSTRUCTION OF SV40 VIRUSES AND
PSEUDOVIRUSES



DECLARATION OF ORLY BEN-NUN-SHAUL, M.Sc., UNDER RULE 132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Orly Ben-Nun-Shaul, of 16 Hamelin Boulevard, Jerusalem, Israel, declare and state that:

1. I received a B.Sc. in Biology from the Hebrew University of Jerusalem in 1986, and a M.Sc. in Microbiology from the Hebrew University in Jerusalem in 1988. Since 1988, I have been working in the Department of Hematology at the Hadassah University Hospital.
2. I have read the above-referenced application "In Vitro Construction of SV40 Viruses and Pseudoviruses" by inventors Ziv Sandalan, Ariella Oppenheim and Amos Oppenheim, U.S.S.N. 09/068,293, filed May 6, 1998.

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3. I am presently working in Dr. Anella Oppenheim's laboratory, and have conducted research with Dr. Oppenheim regarding the subject matter of U.S.S.N. 09/068,293. Discussed below are experiments and results demonstrating that the *ori* sequence is not required for *in vitro* packaging in the constructs described in U.S.S.N. 09/068,293.
4. The packaging experiment was performed with DNA of the plasmid pHAMDB1, which does not contain the SV40 *ori* element, nor any other SV40 sequence (Kane, S.E. *et al.*, 1988, *Mol. Cell Biol.* 8:3316-3321), as illustrated in the attached restriction map. As shown below, the experiment clearly demonstrated that this plasmid was packaged at exactly the same efficiency as a series of plasmids that do contain SV40 *ori*, as illustrated in Table 2 of the patent application (U.S.S.N. 09/068,293).
5. Nuclear extracts of SF9 cells were prepared according to Schreiber (Schreiber, P., *et al.*, 1989, *Nucl. Acids Res.* 15:6419-6436), from cells infected with recombinant baculovirus expressing SV40 VP1. One microliter of nuclear extracts (protein concentration 4.8 µg/µl) was mixed by vortexing with 1 µg DNA in a total volume of 4 µl and placed at 37°C for six hours. CaCl₂ and MgCl₂ were added to final concentrations of 100 µM and 8 mM respectively, in a total volume of 6 µl, and the reactions were incubated for one additional hour on ice. DNase I digestion was performed using 0.5 unit of enzyme for 10 minutes on ice, and stopped by the addition of EDTA to a final concentration of 5 mM.
6. The DNase I treatment was used to remove DNA which was not stably packaged. The reaction products were assayed for infectious units (IU) on CV1-PD monolayers, grown in Dulbecco's modified Eagle's medium with 10% FBS, using a standard SV40 infection protocol (CV1-PD is a sub-line of CV1, the parental line of CMT4). Sub-confluent monolayers were incubated with the packaging mixture for 120 minutes at 37°C, with occasional agitation, followed by the addition of fresh medium. The number of infective centers obtained for a 6 µl reaction mixture was used in computing the titer of IU/ml.
7. The procedure yielded infectious units with pHAMDB1. The experiments showed that packaging pHAMDB1 with nuclear extract containing VP1 yielded 96 infectious

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centers per 6ul reaction, equivalent to 1.6×10^7 infectious units (IU) per ml. Thus, the ori sequence, while convenient for use in the assay system as it facilitates DNA amplification in the infected cells, is not necessary for packaging.

8. I further declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by me are punishable by fine or imprisonment, or both under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Orly Ben-Nun-Shaul
Orly Ben-Nun-Shaul

4.2.01
Date

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proteins

o Rusconi and Walter Schaffner

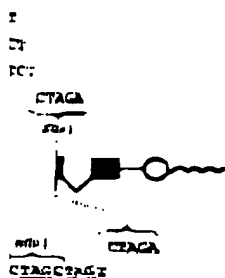
CH-8093 Zurich, Switzerland

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1' — 67-69 nt — cDNA
3' — 99 nt — cDNA

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non codon of HSV TK) is indicated by
the stop codons are underlined, pSP65
40 origin of replication is indicated by

Cambridge Center, Cambridge, MA 02142.

51. (2) Severne, Y. et al. (1988) EMBO

Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells

Edgar Schreiber, Patrick Manthas, Michael M. Müller and Walter Schaffner

Institut für Molekularbiologie II der Universität Zürich, Henggerberg, CH-8093 Zürich, Switzerland
Submitted July 5, 1989

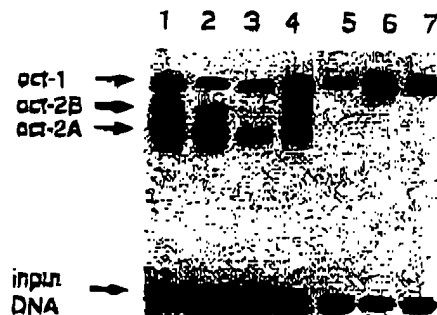
Octamer binding proteins (oct-1, oct-2A/B) are transcription factors which regulate the expression of immunoglobulin heavy chain genes and various housekeeping genes (reviewed in 1). Here we present an extremely simple protocol, modified from that of Lee et al. (2), which allows one to make nuclear extracts from as few as 5×10^4 cells or from lymphocytes isolated from 2 ml of peripheral blood (3). At the same time RNA can be isolated from the cytoplasmic fraction and further analysed, e.g. by Northern blotting. Detection of the DNA binding proteins is performed by bandshift assay. Many different cell lines can be quickly screened for the presence of octamer-binding and other transcription factors. Moreover, "factor-induction" experiments can be conveniently performed in small scale cultures. In addition, this technique will allow clinical investigations using biopsy material or blood mononuclear cells, e.g. lymphocytes purified from 2 ml of peripheral blood (3).

Typically, $0.5 - 1 \times 10^6$ cells from tissue culture, from homogenized mouse spleen (about 0.5 g) or peripheral blood lymphocytes are collected, washed with 10 ml TBS (Tris buffered saline) and pelleted by centrifugation at $1500 \times g$ for 5 min. The pellet is resuspended in 1 ml TBS, transferred into an Eppendorf tube and pelleted again by spinning for 15 sec in a microfuge. TBS is removed and the cell pellet is resuspended in 400 μ l cold buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF) by gentle pipetting in a yellow tip. The cells are allowed to swell on ice for 15 min, after which 25 μ l of a 10% solution of Nonidet NP-40 (Fluka) is added and the tube is vigorously vortexed for 10 sec. The homogenate is centrifuged for 30 sec in a microfuge. The supernatant containing cytoplasm and RNA is transferred to a fresh tube containing 400 μ l buffer B (10 mM Tris pH 7.5; 7 M urea; 1% SDS; 0.3 M NaAc; 20 mM EDTA) and 600 μ l phenol/chloroform (1:1), mixed immediately and stored at -20°C until it is convenient to further purify the RNA according to (4). The nuclear pellet is resuspended in 50 μ l ice-cold buffer C (20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF) and the tube is vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract is centrifuged for 5 min in a microfuge at 4°C and the supernatant (ca. 55 μ l) is frozen in aliquots at -70°C . Usually, 1-2 μ l of this extract (ca. 2-4 μ g protein) is used for a bandshift assay in the presence of 3 μ g poly dI dC as described in (5).

Figure legend: Autoradiography of a bandshift experiment with a radiolabelled octamer probe (5) and various "mini-extracts"; arrows designate nuclear proteins oct-1, oct-2A and oct-2B. lane 1: conventionally prepared nuclear extract (6) of BJA-B lymphocytes; lane 2-7 mini-extracts: lane 2: BJA-B lymphocytes; lane 3: mouse spleen; lane 4: human peripheral lymphocytes; lane 5: HeLa cells; lane 6: HUT 78 T-cells; lane 7: MLA 144 T-cells.

References:

- (1) Schreiber, E., Müller, M.M., Schaffner, W. and Manthas, P. in *Tissue specific gene expression* (Ed. by R. Renkawitz) VCH Weinheim, 1989.
- (2) Lee, K.A.W. et al. (1988) *Gene Anal. Techn.* 5, 22-31.
- (3) Pharmacia data sheet "Ficoll paque", Uppsala Sweden.
- (4) Gough, N.M. (1988), *Anal. Biochem.*, 173, 93-95.
- (5) Schreiber, E. et al. (1988) *EMBO J.*, 7, 4221-4229.
- (6) Dignam, J.D. et al. (1983) *Nucleic Acids Res.*, 11, 1475-1489



Use of a Cloned Multidrug Resistance Gene for Coamplification and Overproduction of Major Excreted Protein, a Transformation-Regulated Secreted Acid Protease

SUSAN E. KANE, BRUCE R. TROEN, SUSANNAH GAL,[†] KAZUMITSU UEDA,[‡] IRA PASTAN,
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Laboratory of Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892

Received 12 November 1987/Accepted 14 April 1988

Malignantly transformed mouse fibroblasts synthesize and secrete large amounts of major excreted protein (MEP), a 39,000-dalton precursor to an acid protease (cathepsin L). To evaluate the possible role of this protease in the transformed phenotype, we transfected cloned genes for mouse or human MEP into mouse NIH 3T3 cells with an expression vector for the dominant, selectable human multidrug resistance (*MDR1*) gene. The cotransfected MEP sequences were efficiently coamplified and transcribed during stepwise selection for multidrug resistance in colchicine. The transfected NIH 3T3 cell lines containing amplified MEP sequences synthesized as much MEP as did Kirsten sarcoma virus-transformed NIH 3T3 cells. The MEP synthesized by cells transfected with the cloned mouse and human MEP genes was also secreted. Elevated synthesis and secretion of MEP by NIH 3T3 cells did not change the nontransformed phenotype of these cells.

MEP, the major excreted protein of transformed mouse cells (10), is the precursor to an acid protease which recently has been identified as cathepsin L (7, 8, 8a, 17, 18). The 39-kilodalton (kDa) mouse MEP is synthesized and secreted in large quantities by malignantly transformed NIH 3T3 cells (9-12) and by phorbol myristate acetate- or platelet-derived growth factor-treated cells (12, 24). Within these cells, the 39-kDa precursor protein is processed to two lower-molecular-mass forms, 29 and 20 kDa, which represent the active forms of the protease in lysosomes (9).

To study the possible function of MEP in growth control and/or transformation of animal cells, it was found necessary to overproduce MEP in otherwise normal cells. We used the human gene (*MDR1*) for multidrug resistance (*MDR*) as a dominant marker for cotransfection and amplification of the cloned mouse and human MEP genes.

The *MDR1* gene is overexpressed and frequently amplified in various rodent and human cell lines which express simultaneous resistance to drugs such as colchicine, vinblastine, adriamycin, and actinomycin D (6, 22, 25). Resistance is due to the action of the *MDR1* gene product, a 170-kDa, energy-dependent drug efflux pump (22) termed P-glycoprotein (13). A retroviral expression vector containing a full-length cDNA clone of the human *MDR1* gene (*pHMDR1*) has been shown to confer *MDR* when transfected into NIH 3T3 cells (32). We demonstrate here that *pHMDR1* can be used as a dominant selectable marker to cotransfect the MEP gene into NIH 3T3 cells. By selection of transfected cells with increasingly higher concentrations of drug, the *MDR1* DNA and cotransfected MEP sequences are amplified, and MEP is overexpressed.

By amplifying the MEP gene with the *MDR* selection system, we found it possible to achieve levels of expression

and secretion of MEP comparable to those found in cells malignantly transformed by Kirsten sarcoma virus (KNH cells). These high levels of MEP had no effect on cell morphology, growth rate, growth in soft agar, growth in low serum, or tumorigenicity in nude mice, suggesting that overexpression and secretion of MEP are not sufficient to mimic any of these aspects of the transformed phenotype.

MATERIALS AND METHODS

Cells. NIH 3T3 cells and KNH cells were kindly provided by C. Scher (University of Pennsylvania School of Medicine). All cell lines were maintained in Dulbecco modified Eagle medium containing 10% calf serum (Colorado Serum Co.), 5 mM glutamine, 50 U of penicillin per ml, and 50 µg of streptomycin per ml. Colchicine was obtained from Sigma Chemical Co. and diluted from dimethyl sulfoxide stock solutions of 10 mg/ml to appropriate concentrations in complete medium.

Plasmids. *pHMDR1* (previously designated *pHamDR* [32]) was constructed as follows. A 4,380-base-pair *SacI*-*EcoRI* fragment of a human *MDR1* cDNA (32) was modified at its 5' end with a *SacII* linker and at its 3' end with an *XhoI* linker. This fragment was inserted into the *SacII*-*XhoI* site of the eucaryotic expression vector *pCOL* (provided by D. Lowy) to yield *pHMDR1*, with the human *MDR1* cDNA sequences inserted between the two long terminal repeats of Harvey murine sarcoma virus. *pcosMEP5* is a genomic clone of the mouse MEP gene in the cosmid vector *pSV13* (19, 30). *pHul6* is a cDNA clone of the human MEP sequences in the Okayama-Berg expression vector (8a, 21). DNAs for transfection were isolated from *Escherichia coli* by standard procedures and purified by two consecutive centrifugations to equilibrium in cesium chloride-ethidium bromide gradients.

Cell transfection and colchicine selection. NIH 3T3 cells were transfected by the calcium phosphate coprecipitation method as described previously (26) with *pHMDR1*+*pcosMEP5*, *pHMDR1*+*pHul6*, or *pHMDR1* only. Control cells received no DNA. Cells were washed after 16 h, and 24 h later they were trypsinized and replated in medium con-

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taining 60 ng of colchicine per ml. After 10 days in selective medium, colonies were pooled into either 40 or 80 ng of colchicine per ml and allowed to grow for at least 10 days. At the same time, individual colonies were picked and grown as clones in 80 ng of colchicine per ml. Both the pooled populations and clones were selected in approximately two-fold increments of colchicine concentration up to 1.5 μ g/ml (populations) or 1 μ g/ml (clones). At each stage of the selection, cells were allowed to grow in the appropriate drug concentration for at least 5 to 10 days before being plated at the next higher step.

Labeling and immunoprecipitation. Transfected cells were labeled with 50 μ Ci of [35 S]methionine (Amersham Corp.) per ml in Dulbecco-Vogt medium lacking methionine (National Institutes of Health Media Unit) and supplemented with 5 mM glutamine, 50 U of penicillin per ml, and 50 μ g of streptomycin per ml. Labeling was done for 3 h, and labeled culture medium and cell lysates were collected as detailed previously (9). Immunoprecipitations were performed as described previously with either a rabbit anti-mouse MEP antibody to precipitate mouse MEP (11) or a rabbit anti-cathepsin L antibody (a gift from K. W. Mason) to precipitate human MEP. For immunoprecipitations of mouse MEP, 5×10^5 trichloroacetic acid-precipitable counts were used, for immunoprecipitations of human MEP, 5×10^6 cpm were used. Immunoprecipitates were run on a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel as described by Laemmli (15). The gel was treated with 2,5-diphenyloxazole in dimethyl sulfoxide (2), and fluorography was performed at -70°C .

DNA isolation and Southern analysis. Genomic DNA was isolated from transfected or control cells essentially as described previously (26). After brief sonication, DNA was digested to completion with *Eco*RI, separated on an 0.8% agarose gel, and transferred to nitrocellulose by the method of Southern (27). Hybridizations were performed at 42°C in 50% formamide- $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl) plus 15 mM sodium citrate (pH 7.0)-10 \times Denhardt solution (1 \times Denhardt solution is 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll)-50 mM Tris (pH 7.5)-0.1% SDS, 100 μ g of single-stranded DNA per ml, and 10^7 dpm of the appropriate probe. Washes were done first with $2\times$ SSC-0.2% SDS at 60°C and then with $0.1\times$ SSC-0.2% SDS at 60°C . The probe for MDR-specific sequences was an internal 3.4-kilobase *Eco*RI fragment isolated from pHaMDR1 and labeled with 32 P by nick translation (Lofstrand Labs). The mouse MEP probe was an *Eco*RI-*Pst*I fragment derived from the 3' end of the MEP-coding region of pcusMEP5 (30) and contained some downstream noncoding sequences as well. The MEP probe was labeled with 32 P by the random oligonucleotide primer method (Lofstrand Labs). Washed filters were exposed to X-Omat AR film (Eastman Kodak Co.) at -70°C with an intensifying screen.

RNA isolation and RNase protection. Total cell RNA was isolated from transfectants by the guanidine thiocyanate method of Chirgwin et al. (5). For RNase protection analysis, antisense transcripts were synthesized with, as a template, a 110-base-pair *Sau*3AI fragment from a mouse MEP cDNA clone (nucleotides 122 to 232 on the cDNA map (31)) inserted into pGEM-3 (Promega Biotec); the plasmid was linearized at the *Pvu*II site of the pGEM-3 vector. The resulting probe, synthesized from the SP6 promoter of pGEM-3, contained 110 nucleotides complementary to mouse MEP mRNA. The RNase protection assays were performed as described by Melton et al. (20) with 10 μ g of total cell mRNA and 1.5×10^5 to 3×10^5 cpm of antisense

probe. Final reaction products were resolved on a 6% polyacrylamide gel containing 8 M urea and exposed to X-Omat AR film at -70°C with an intensifying screen.

RESULTS

Cotransfection and amplification of MDR and MEP. Plasmid pHaMDR1 contains a full-length cDNA clone of the human *MDR1* gene inserted between two Harvey murine sarcoma virus long terminal repeats. This plasmid was previously shown to confer MDR when transfected into NIH 3T3 cells (32).

The cosmid pcusMEP5 is a genomic clone of the mouse MEP (mouse cathepsin L) gene, while pHu16 is a full-length cDNA clone of the human MEP (human cathepsin L) homolog in the Okayama-Berg expression vector. The mouse genomic clone expresses the full 39-kDa precursor form of MEP when transfected into CV-1 or A431 cells (30). This protein is secreted and processed normally by the transfected cells and is enzymatically active. Likewise, the human cDNA clone expresses in transfected NIH 3T3 cells a 42-kDa protein which comigrates on SDS-polyacrylamide gels with the human homolog of MEP from A431 cells (data not shown).

We used *MDR1* as a selectable marker in cotransfections with either the mouse or human MEP clone. NIH 3T3 cells were transfected with pHaMDR1+pcusMEP5 DNA, with pHaMDR1+pHu16 DNA, or with pHaMDR1 DNA only as described in Materials and Methods. Transfected cells were initially selected in medium containing 60 ng of colchicine per ml, a drug concentration at which mock-transfected cells yielded no background colonies. Transfections with plasmid pHaMDR1 resulted in colchicine-resistant colonies at a frequency of 5×10^{-4} to 2×10^{-3} . After 10 days in selective medium, 100 to 300 colchicine-resistant colonies were pooled into either 40 or 80 ng of colchicine per ml and allowed to grow in culture for at least 10 days. For some of the experiments described below, individual colonies of transfected cells were picked and grown initially in 80 ng of colchicine per ml. To amplify the transfected *MDR1* sequences, we selected cells with increasing concentrations of colchicine in the culture medium (see Materials and Methods).

To analyze MEP expression in transfected cells, pooled colonies growing in the presence of 40, 160, and 640 ng and 1 μ g of colchicine per ml were labeled metabolically with [35 S]methionine, and immunoprecipitations were performed on cell lysates and culture medium. The results (Fig. 1) indicated that colchicine-resistant cells also expressed the cotransfected MEP sequences. (In the case of the pHaMDR1+pcusMEP5 transfections, protein made from the MEP cosmid comigrated with the low level of endogenous MEP produced by NIH 3T3 cells.) Furthermore, as colchicine resistance increased, MEP synthesis also was amplified. MEP synthesized by transfected cells was compared with MEP produced by K18H cells, derivatives of NIH 3T3 cells transformed by Kirsten sarcoma virus (Fig. 1, lane 1). K18H cells synthesize about 50-fold more MEP than do NIH 3T3 cells and secrete 50 to 60% of the 39-kDa form of this protein into the culture medium. In the presence of 1 μ g of colchicine per ml, the pHaMDR1+pcusMEP5 cell line synthesized and secreted at least as much MEP as did K18H cells.

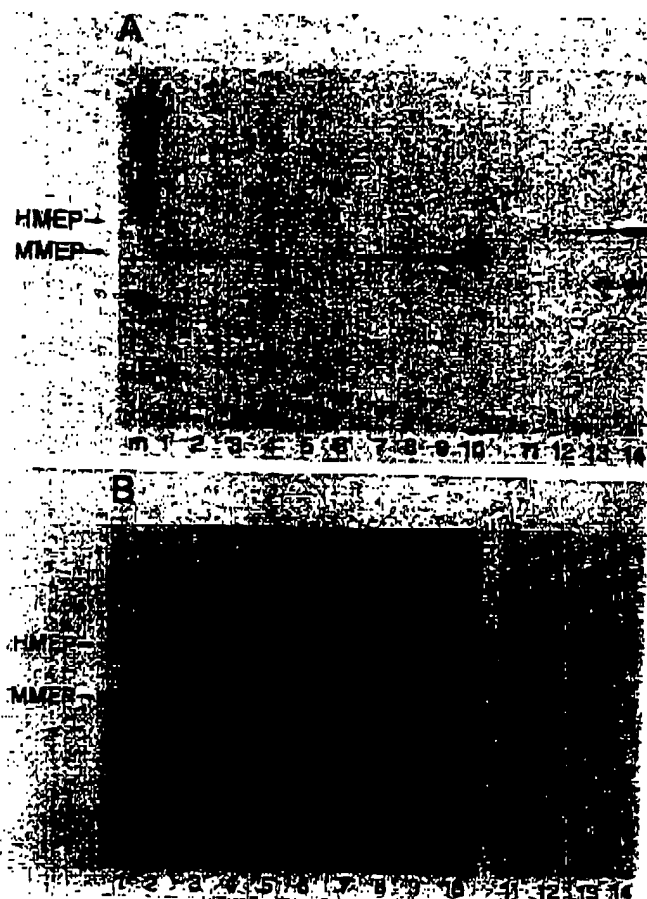


FIG. 1. MEP in colchicine-selected transfections. Populations of transfectants containing pHaMDR1 DNA alone, pHaMDR1+pcosMEP5 DNA, or pHaMDR1+pHul6 DNA were labeled with [35 S]methionine for 3 h. Cell lysates (A) or culture supernatants (B) were then immunoprecipitated with anti-MEP antibody as described in Materials and Methods. Pictured are fluorograms of the resulting SDS-polyacrylamide gels. (A) Immunoprecipitation of cell lysates of KNIH cells (lane 1); NIH 3T3 cells (lane 2); pHaMDR1 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml (lanes 3 to 6, respectively); pHaMDR1+pcosMEP5 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml (lanes 7 to 10, respectively); and pHaMDR1+pHul6 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml (lanes 11 to 14, respectively). For lanes 1 to 10, precipitations were performed with α -mouse MEP antibody and 5×10^5 trichloroacetic acid-precipitable counts of lysate; for lanes 11 to 14, precipitations were performed with α -human cathepsin L antibody and 5×10^4 cpm of lysate. m, Protein molecular mass standards of 68, 43, 25.7, and 18.4 kDa, from top to bottom. (B) Immunoprecipitation of culture medium of labeled cells. Lanes 1 and 2 are NIH 3T3 and KNIH cells, respectively. For all other lanes, antibodies and counts are as described for panel A.

Interestingly, secretion of both the transfected mouse and transfected human proteins also increased with higher colchicine resistance (Fig. 1B), suggesting that secretion of MEP by growth-stimulated NIH 3T3 cells or by KNIH cells could simply be a result of overexpression of the lysosomal protein rather than a secondary effect of growth stimulation or transformation.

Amplification of MEP sequences. To study the mechanism of increased drug resistance and increased MEP synthesis in transfected cells, we analyzed RNA and DNA from these cell lines. Steady-state MEP mRNA levels in transfected populations were measured by RNase protection analysis

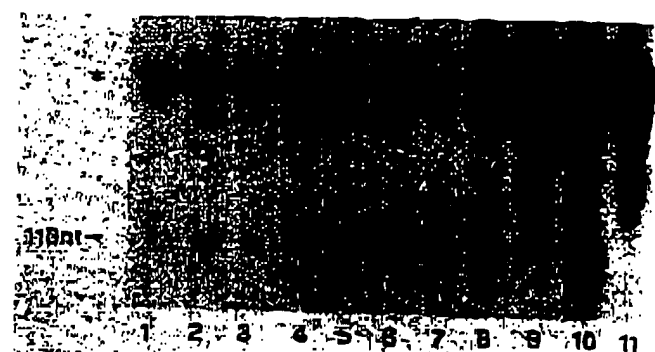


FIG. 2. RNase protection analysis. Total cell RNA was isolated from populations of pHaMDR1 or pHaMDR1+pcosMEP5 transfectants by the method of Chirgwin et al. (5). RNase protection was performed with 10 μ g of RNA and an antisense RNA probe which protects 110 nucleotides of internal MEP coding sequences within both the transfected MEP mRNA and the endogenous mouse MEP mRNA of NIH 3T3 cells (see Materials and Methods). Illustrated is an autoradiogram of the RNase reaction products separated on a 6% polyacrylamide gel. Lanes: 1 to 4, RNA from pHaMDR1 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml, respectively; 5 to 8, RNA from pHaMDR1+pcosMEP5 transfectants at 40, 160, and 640 ng and 1 μ g of colchicine per ml, respectively; 9, RNA from NIH 3T3 cells; 10, RNA from KNIH cells; 11, no RNA. The position of 110 nucleotides (110nt) band is indicated on the left. The asterisk represents undigested probe (for lanes 9 to 11, probes for both mouse MEP and human MEP [data not shown] were used, accounting for the more intense band of undigested probe; the human MEP probe does not cross-react with mouse MEP mRNA in NIH 3T3 cells.)

(see Materials and Methods). Figure 2 shows that MEP mRNA increased with increasing colchicine resistance in pHaMDR1+pcosMEP5 transfectants (Fig. 2, lanes 5 to 8). This increase paralleled the protein enrichments during selection. MEP mRNA in control pHaMDR1 transfectants (Fig. 2, lanes 1 to 4) remained constant at the low endogenous levels normally seen in NIH 3T3 cells (lane 9). Similar results were obtained for the pHaMDR1+pHul6 transfectants (data not shown).

To determine whether increased MDR and MEP expression was accompanied by amplification of the respective DNAs, we picked individual clones from the initial transfections with pHaMDR1, pHaMDR1+pcosMEP5, or pHaMDR1+pHul6. Each clone was subsequently grown in stepwise increments of colchicine as described above, and genomic DNA was isolated from cells growing in the presence of 80, 160, and 640 ng and 1 μ g of colchicine per ml. DNA was digested with *EcoRI*, separated on an 0.8% agarose gel, and transferred to nitrocellulose filters. Filters were hybridized with probes specific for either *MDR1* or MEP. The results for one clone from the mouse MEP transfection are shown in Fig. 3. *MDR1* and MEP sequences were amplified in parallel in the transfectants as colchicine resistance increased.

Effect of MEP overexpression. MEP expression and secretion are stimulated in transformed NIH 3T3 cells and in cells treated with tumor promoters or growth factors. MEP might therefore be a primary stimulator of cell growth and/or a primary cause of the transformed state. To investigate this possibility, we analyzed the phenotypes of populations of transfected cells which overexpressed MEP in the absence of any other transformation or growth stimulus.

pHaMDR1+pcosMEP5 cells resistant to 1 μ g of colchicine per ml produced almost 20-fold more MEP than did

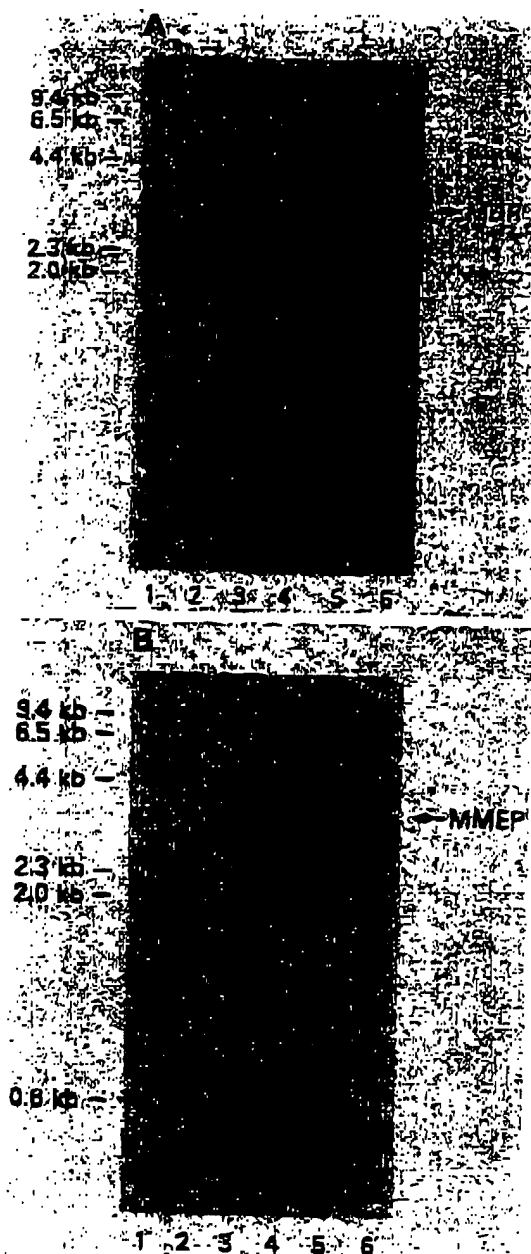


FIG. 3. Southern analysis of genomic DNAs. Genomic DNA was isolated from a cloned cell line of pHaMDR1+pcosMEP5 transfectants growing in the presence of 80, 160, and 640 ng and 1 µg of colchicine per ml. DNA was digested with *Eco*RI, separated on an 0.8% agarose gel, and transferred to nitrocellulose. Shown are the autoradiograms of the resulting Southern analyses (see Materials and Methods). (A) A filter was hybridized with a 3.4-kb *Eco*RI fragment isolated from the MDR1 coding region of pHaMDR1. (B) A duplicate filter was hybridized with a mouse MEP-specific probe which detects an MEP-pSV13 junction fragment from the transfected cosmid pcosMEP5A (MMEP). The probe also hybridized with the single-copy endogenous mouse MEP gene, indicated by the asterisk. Lanes: 1 and 2, NIH 3T3 and KNIH cells, respectively. 3 to 6, pHaMDR1+pcosMEP5 clone at 80, 160, and 640 ng and 1 µg of colchicine per ml, respectively. Positions of DNA standards are indicated to the left of each panel. Multiple bands are due to multiple integration sites of the transfected DNA.

TABLE 1. Transformation assays

Cell line	Colonies in soft agar ^a	Growth in 0.5% serum (%) ^b	Tumorigenicity ^c
pHaMDR1+pcosMEP5	0	0	-
pHaMDR1+pHu16	0	0	ND
pHaMDR1	0	0	-
NIH 3T3	0	0	-
KNIH	250	82	+
K-MDR	155	59	+

^a Duplicate 10-cm dishes were seeded with 600 cells in 0.35% agar onto a feeder layer of 0.5% agar. Values are the averages of the number of colonies formed after 10 days.

^b Growth in complete medium containing 0.5% calf serum was determined as the plating efficiency relative to 100% efficiency in 10% calf serum. Values are the averages of duplicate assays.

^c Nude mice were injected subcutaneously with transfected or control populations of cells and monitored for tumor formation. Results for each cell line are presented as tumors detected (+) or not detected (-) 19 days after injection of 10^6 cells into each of three mice. Delayed tumor growth (greater than 3 weeks) was detected in some of the mice injected with 1×10^6 or 5×10^6 cells of the pHaMDR1 or NIH 3T3 populations, but no tumors were seen with the NIH 3T3 cells transfected with pHaMDR1+pcosMEP5 up to 5 weeks after injection of 5×10^6 cells. ND, Not done.

pHaMDR1 transfectants with the same drug resistance, a level of MEP nearly comparable to that in transformed KNIH cells. pHaMDR1+pHu16 cells grown in the presence of 1 µg of colchicine per ml expressed about 10-fold more MEP than did the corresponding transfectants grown at 40 ng of colchicine per ml. Populations of these MEP cells were analyzed for cell morphology, growth rate, growth in soft agar, growth in low serum, and tumorigenicity relative to control cells.

As a control for the effect of colchicine or MDR1 expression in these experiments, KNIH cells were transfected with the MDR1 gene and selected stepwise with colchicine up to 1 µg/ml as described above. The resulting cells were designated K-MDR cells. Both KNIH and K-MDR cells exhibited a more rounded shape than did NIH 3T3 cells and were not contact inhibited in culture. They grew about twice as fast as NIH 3T3 cells, readily formed colonies in soft agar, were able to grow in low serum, and caused tumors when injected into nude mice.

The transfected NIH 3T3 cells producing similar amounts of MEP as KNIH cells appeared morphologically normal and grew at a rate similar to that of control cells transfected with pHaMDR1 (data not shown).

The results of three assays of malignant transformation are summarized in Table 1. Growth in soft agar was used to determine whether MEP overexpression could confer anchorage independence on NIH 3T3 cells. Whereas KNIH and K-MDR cells formed colonies in soft agar after 7 to 10 days, we saw no colonies with the MDR1-MEP cell lines or with the control NIH 3T3 or MDR1 cells. Growth in low serum was assayed by plating cells in medium containing 10, 5, 2, 1, 0.2, or 0.1% serum. The relative cloning efficiencies of the cell lines indicated that KNIH and K-MDR cells were able to grow in medium containing only 0.5% serum, while NIH 3T3 cells and all of the transfected populations required 1 to 2% serum. Finally, cells synthesizing and secreting MEP were not tumorigenic in nude mice, even at the highest dose of injected cells (5×10^6 cells per mouse), at least 4 weeks after injection. KNIH and K-MDR cells caused tumors within 1 week at doses that were 5- to 10-fold lower.

DISCUSSION

We now report that the *MDR1* gene is an excellent dominant, selectable marker in NIH 3T3 cells. The pHaMDR1 expression vector has been transfected into a variety of other rodent and human cell lines and confers drug resistance with good efficiency (unpublished results). In addition, the *MDR1* gene has the advantage of being easily amplified in these cell lines, with a variety of drugs as the selective agents. Other such selectable, amplifiable markers include the dihydrofolate reductase gene (1, 28), the *E. coli* gene for asparagine synthetase (3), the adenosine deaminase gene (14), and the ornithine decarboxylase gene (4).

Further, we have used the human *MDR1* gene as a means of cotransfecting the nonselectable MEP gene into NIH 3T3 cells. Probably by virtue of its coinTEGRATION along with *MDR1* DNA into the host genome, MEP DNA was also cotransfected upon selection with progressively increasing concentrations of colchicine in the culture medium. We were able to attain very high expression of MEP in otherwise normal cells, reaching MEP levels comparable to those seen in transformed KN1H cells.

The overproduction of MEP led to the increased secretion of the protein by the transfected cells, suggesting that the secretion of MEP by KN1H cells is not a secondary effect of the transformed state of those cells but results directly from the presence of large amounts of the lysosomal protein. The effect on the lysosomal or secretory pathway is a specific one, however, since other lysosomal proteins are not coordinately secreted along with MEP (23). This phenomenon is similar to that observed with carboxypeptidase Y in yeast (29). These results suggest that there might be specific, saturable receptors for proteins in the sorting and transport machinery of cells.

Transfected cells which expressed and secreted large amounts of MEP appeared to be phenotypically normal in several transformation assays. When the population of pHaMDR1+pcosMEP5 transfectants growing in the presence of 1 μ g of colchicine per ml was subcloned, 8 of 10 individual clones expressed levels of MEP at least 20-fold higher than those in control cells (unpublished results), suggesting that we should have been able to detect any effects of MEP on transformation in our assays with cell populations. Thus, MEP overexpression and secretion are not sufficient for inducing the transformed state of NIH 3T3 cells. However, MEP might be involved in malignancy in other ways. If it is not a primary agent in transformation or growth control, it might act in coordination with other factors, such as the *c-myc* or adenovirus E1a genes (16), to elicit a growth response.

With the ability to express very high levels of MEP without requiring cell transformation or stimulation by growth factors, we are in a position to understand the possible function of this lysosomal protease in growth control and the transformed phenotype. The *MDR* system, which appears to serve as a strong dominant marker which can be easily amplified in many cell types, should be useful for these studies and others requiring high-level gene expression in transfected cells.

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